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Alternate steroid sulfation pathways targeted by LC-MS/MS analysis of disulfates. Application to prenatal diagnosis of steroid synthesis disorders

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Short title

Steroid disulfates in prenatal diagnosis

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ABSTRACT

The steroid disulfates (*aka* bis-sulfates or bis(sulfates)) are a significant but minor fraction of the urinary steroid metabolome that have not been widely studied because major components are not hydrolyzed by the commercial sulfatases commonly used in steroid metabolomics. In early studies, conjugate fractionation followed by hydrolysis using acidified solvent (solvolysis) was used for the indirect detection of this fraction by GC-MS. This paper describes the application of a specific LC-MS/MS method for the direct identification of disulfates in urine, and their use as markers for the prenatal diagnosis of disorders causing reduced estriol production: STSD (Steroid Sulfatase Deficiency), SLOS (Smith-Lemli-Opitz Syndrome) and PORD (P450 Oxido-Reductase Deficiency). Disulfates were detected by monitoring a constant-ion-loss (CIL) from the molecular di-anion. While focused on disulfates, our methodology included an analysis of intact steroid glucuronides and monosulfates because steroidogenic disorder diagnosis usually requires an examination of the complete steroid profile. In the disorders studied, a few individual steroids (as disulfates) were found particularly informative: pregn-5-ene-3β,20*S*-diol, pregn-5-ene-3β,21-diol (STSD, neonatal PORD) and 5α-pregnane-3β,20*S*-diol (pregnancy PORD). Authentic steroid disulfates were synthesized for use in this study as aid to characterization. Tentative identification of 5ξ-pregn-7-ene-3ξ,20*S*-diol and 5ξ-pregn-7-ene-3ξ,17,20*S*-triol disulfates was also obtained in samples from SLOS affected pregnancies. Seven ratios between the detected metabolites were applied to distinguish the three selected disorders from control samples. Our results show the potential of the direct detection of steroid conjugates in the diagnosis of pathologies related with steroid biosynthesis.

INTRODUCTION

From the earliest days of steroid metabolomics, the principal conjugated forms of steroids (sulfates and glucuronides) have been hydrolyzed prior to analysis, and for decades the instrument of choice for steroid separation and measurement has been GC-MS (Shackleton and Marcos 2006). While this technique remains the gold-standard for steroid profiling, LC-MS/MS has been increasingly adopted because of the simplified sample preparation and speed of analysis, mainly provided by absence of a derivatization step. This is in spite of the poor ionization for fully reduced steroids by electrospray (ESI) (Pozo, et al. 2007). While an advance, this methodology still retains the most time-consuming step of sample preparation, the enzymatic or chemical hydrolysis of conjugates (Gomes, et al. 2009). Hydrolysis itself can take several hours and requires a further solid phase extraction (SPE). Necessary chemical derivatization for GC-MS can also take hours.

Intact steroid conjugates have been analyzed by mass spectrometry since the introduction of particle beam ionization (e.g. Fast Atom Bombardment, FAB) in the 1980s (Shackleton and Straub 1982; Shackleton 1983). Their spectra have dominant deprotonated molecules $[M-H]^-$ in negative ion mode allowing ease of mass determination. Conjugate analysis was simplified with the introduction of electrospray ionization (ESI) and incorporation of HPLC and MS/MS. Glucuronides can be analyzed in both positive and negative ionization modes by monitoring $[M+NH_4]^+$ and $[M-H]^-$ respectively (Fabregat, et al. 2013). In the case of monosulfates, collision-induced-dissociation (CID) of the strong $[M-H]^-$ ions shows a distinctive hydrogen sulfate (HSO_4^-) fragment at *m/z* 97 (Shackleton 1983; Galuska, et al. 2013). Direct detection of steroid conjugates also circumvents the ionization problems of reduced steroids (Pozo, et al. 2007) as phase II metabolites have readily ionized functionality (i.e. a carboxylic acid in glucuronides and an acidic sulfate ester in sulfates).

While mono-conjugates dominate the sulfate fraction of urinary steroids, it has been known since the 1960s that disulfates (diS, also referred to as bis(sulfates) or bis-sulfates to distinguish them from compounds containing the disulfate ($S_2O_7^{2-}$) unit) are significant components of the metabolome (Pasqualini and Jayle 1962; Arcos and Lieberman 1967; Shackleton, et al. 1968a; Shackleton, et al. 1968b; Jänne, et al. 1969). Early studies by GC-MS of separated conjugate fractions showed that, in addition to the classic 3β-sulfated steroids, hydroxyls at positions 16β-, 17-(α and β) and 18- in androgens and 20- and 21- in pregnanes were prone to sulfation (Jänne, et al. 1969; Jänne and Vihko 1970; Laatikainen, et al, 1972; Meng and Sjövall 1997).

Since these original studies, disulfates have been a largely ignored component of the metabolome that nevertheless had significant potential to expand the understanding of steroid biosynthetic and metabolic pathways. Given this, we sought to develop LC-MS/MS methodology to target this group. It was found that constant-ion-loss (CIL) of hydrogen sulfate (HSO_4^-) fragment at *m/z* 97 from the molecular di-anion $[M-2H]^{2-}$ was the most useful reaction to monitor (McLeod, et al. 2017).

The ease of steroid disulfate analysis led us to investigate their use in diagnosis of steroid biosynthetic disorders. One particular area of interest to the authors has been the pre-natal diagnosis of single-gene disorders of estriol (E3) synthesis by urine analysis, of which we have studied three conditions by GC-MS, viz., Steroid Sulfatase Deficiency (STSD), Smith-Lemli-Opitz Syndrome (SLOS, 7-dehydrosterol reductase deficiency) and cytochrome P450 Oxido-Reductase Deficiency (PORD) (Marcos et al. 2009; Shackleton, et al. 2004a; Shackleton et al. 2004b; Arlt et al. 2004; Reisch, et al. 2013; Shackleton et al. 2007). This communication offers our preliminary observations of the disulfated steroids excreted in these disorders at around mid-pregnancy. While focusing on disulfates, selected monosulfates and

glucuronides were also included; evaluating the complete steroid profile is crucial to diagnosing aberrant steroid biosynthesis (Shackleton and Marcos 2006).

MATERIALS AND METHODS

Reagents and chemicals

Steroid starting materials were obtained from Steraloids (Newport, RI, USA). Chemicals and solvents including sulfur trioxide pyridine complex (SO_3.py), *N*,*N*-dimethylformamide (DMF) and ammonium formate (HPLC grade) were purchased from Sigma–Aldrich (St Louis, MO, USA). Aqueous ammonia solution (25%), and acetonitrile and formic acid (LC-MS grade) were from Merck (Darmstadt, Germany). MilliQ water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Synthesis of reference steroid disulfates

The qualitative synthesis of steroid disulfates as the ammonium salts was performed as previously described (McLeod, et al. 2017) with small modifications. Briefly, 1 mg of each steroid standard was directly dissolved in a freshly prepared solution of SO_3.py complex (20 mg, 124 µmol, ~38 eq/steroid or ~19eq/hydroxyl group) in DMF (100 µL) and incubated at room temperature for 72 hours. The success of synthesis was confirmed by analysis of reaction using both LC-MS in scan mode and LC-MS/MS for collision induced dissociation studies. The purification of synthesised disulfates was performed using SPE as previously described (McLeod et al, 2017).

Steroid disulfate reference materials isolated as the corresponding ammonium salts and used in this study included: 5α-pregnane-3β,20*S*-diol bis(sulfate), (3β5αPD-diS); 3β,21-dihydroxypregn-5-en-20-one bis(sulfate), (21-hydroxypregnenolone bis(sulfate), 21OHPreg-diS); androst-5-ene-3β,17α-diol bis(sulfate), (5AD(17α)-diS); androst-5-ene-3β,17β-diol bis(sulfate), (5AD(17β)-diS); 3β,16α-dihydroxyandrost-5-en-17-one bis(sulfate), (16α-hydroxydehydroepiandrosterone bis(sulfate),

16αOHDHEA-diS); 3β,16β-dihydroxyandrost-5-en-17-one bis(sulfate) (16β-hydroxydehydroepiandrosterone bis(sulfate), 16βOHDHEA-diS); pregn-5-ene-3β,17α,20*S*-triol 3,20 bis(sulfate), (5PT-diS); pregn-5-ene-3α,20*S*-diol bis(sulfate), (5PD-diS); 5β-pregnane-3β,20*S*-diol bis(sulfate),; 5β-pregnane-3α,20*S*-diol bis(sulfate),; 5α-pregnane-3α,20*S*-diol bis(sulfate),; 5β-pregnane-3β,20*R*-diol bis(sulfate), 5α-pregnane-3β,20*R*-diol bis(sulfate),; 5β-pregnane-3α,20*R*-diol bis(sulfate),; In this manuscript the IUPAC terms for the 20-hydroxypregnane diastereomers are used, S and R, in some publications often trivialized to α and β, respectively.

Two reference materials (3β5αPD-diS and 21OHPreg-diS), were prepared on larger scale and subjected to characterisation by spectroscopic methods. Experimental details and characterization data for these new compounds, together with copies of the ^{1}H NMR, ^{13}C NMR, and ESI LRMS spectra are available from the authors (MM).

Urine Samples

One of our laboratories (Children's Hospital Oakland, Dr. Cedric Shackleton) has been the recipient for urine samples from patients with suspected abnormal steroidogenesis in an attempt to characterize the defects. The studies were approved by the Children's Hospital Institutional Review Board (IBR#2010-038)). Many of the samples used in this study were remnants of those sent to the laboratory for investigation of low pregnancy estriol (generally defined as individuals with serum unconjugated estriol < 0.3 MoM, multiples of median). Other samples were from women who had had a previously affected SLOS child or other symptomatic reasons for concern regarding steroidogenesis. The samples have generally been collected between week 16 and 30 of gestation. They have been stored frozen at -20 °C. Eleven STSD samples were analyzed, and six samples from SLOS affected pregnancies. The neonatal PORD samples were collected at 7, 18 and

23 days. Urine samples from unaffected pregnancies were from a collection held by IMIM (Institut Hospital del Mar d'Investigacions Mèdiques, Barcelona). Normal neonatal urine specimens were from a control urine collection at the Institute of Metabolism and Systems Research (IMSR), University of Birmingham UK.

Sample treatment

Urine extraction was by C18 SPE. Generally, a 2 mL aliquot of urine was passed through a pre-conditioned cartridge. After a washing step with 3 mL water, steroid conjugate analytes were eluted using 2 mL of methanol. After evaporation of a 200 µL aliquot of the elution solvent, the extract was reconstituted in 100 µL of water and 5 µL was injected into the UHPLC-MS/MS system. Stably labelled 17-S$\{^{18}O\}_3$-5α-androstane-3α,17β-diol 3,17-bis(sulfate) and 17-S$\{^{18}O\}_3$-5α-androstane-3β,17β-diol bis (sulfate) were used as internal standards. The labeled sulfate residue was introduced to the steroidal diol mono-sulfate using labelled S$\{^{18}O\}_3$.py generated in situ from labelled sulfuric acid (95% atom) and acetic anhydride in pyridine. Experimental details and characterization data for these internal standards, together with copies of the ^{1}H NMR, ^{13}C NMR, and ESI LRMS spectra are available from the authors (MM)

UHPLC-MS/MS analysis

Disulfates

The study was carried out using a triple quadrupole (XEVO TQ-S micro) mass spectrometer equipped with an ESI source and interfaced to an Acquity UPLC system

for the chromatographic separation (all from Waters Associates, Milford, MA, USA). Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h, and the cone gas flow was 50 L/h. A cone voltage of 30 V and a capillary voltage of 0.4 kV were used in negative ionization mode. The nitrogen desolvation temperature was set to 600 °C, and the source temperature was 150 °C.

The UHPLC separation was performed using an Acquity UPLC CSH Phenyl-Hexyl column (2.1 × 100 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300 µL/min. Water and acetonitrile:water (9:1) both with formic acid (0.01% v/v) and ammonium formate (25 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min, 15%; 0.5 min, 15%; 25 min, 30%; 26 min, 100%; 27 min, 100%; 28 min, 15%; 30 min, 15%. The total analysis time was 30 min.

For the constant ion loss (CIL) scan, dwell times of 6 ms and collision energies of 15 eV were selected for each ion transition. Due to the molecular masses of steroid hormones and metabolites (250-400 Da), the precursor ions of disulfates ($[M\text{-}2H]^{2-}$) were restricted to the range from *m/z* 199 to *m/z* 274. A Selected Reaction Monitoring (SRM) approach containing 75 preselected transitions was used for the simultaneous detection of steroid disulfates. Among them, the transition 228→359 corresponded to the internal standards used in the analysis.

Monoconjugates

While the focus has been on steroid disulfates we have acquired data on steroid monosulfates and glucuronides previously reported as relevant for the studied disorders. Based on previous studies (Gomez, et al. 2014) the product ions at *m/z* 97 and *m/z* 75 for sulfates and glucuronides respectively were chosen (Table 1).

Exceptions were estriol conjugates due to the influence of the aromatic ring. The

neutral loss of the conjugate (80 Da and 176 Da for sulfates and glucuronides respectively were detected).

Quantification

For this study, accurate quantitative measurements have not been conducted for two reasons: 1) lack of some authentic compounds prevented the determination of relative responses of analyte transitions to internal standard transitions; 2) the urine samples were random “spot” collections and not accurate 24 h collections. Instead, we have determined “diagnostic-ratios” from raw mass spectrometric transition responses. These ratios are of an analyte known to be *overproduced* to one known to be *underproduced* in a particular disorder. Such ratios have long been used in GC-MS analysis (Shackleton and Marcos,2006).

RESULTS AND DISCUSSION

Method development

This communication applies recent LC-MS/MS studies on steroid disulfate analysis using the constant-ion-loss (CIL) from the di-anionic precursor $[M\text{-}2H]^{2-}$ (McLeod, et al. 2017). The method was developed for untargeted detection, and designed for the analysis of a maximum number of natural disulfates. The use of this precursor ion and the fact that the product ion has a higher *m/z* value is unusual for small molecules. Determination of disulfates under these conditions gives clean chromatograms and the main interferences observed in the chromatograms are due to the relatively high natural abundance of the ^{34}S isotope (4.25%). The transition coming from the *m/z* 97 loss from an unsaturated (Δ^4, Δ^5, etc.) $\{^{34}S\}_1$-disulfate isotope is completely indistinguishable from the one coming from an A-ring reduced steroid disulfate.

To maximize isobaric steroid metabolite separation (e.g. pregnenediol-diS, the pregnanediol-disulfates and the androstenediol-disulfates) in this study, a phenyl-hexyl column with a relatively high amount of ammonium formate (25 mM) was required to obtain sharp and well resolved chromatographic peaks. Column temperature was critical for this purpose with 30 °C determined as optimum. Under these conditions, a 25 min gradient from 15% to 30% of organic solvent provided desired separation (Figure 1A).

Under optimized conditions the elution order of disulfates was dihydroxyandrostanones < dihydroxypregnanones < androstenediols < pregnanediols. In a specific group, 17β hydroxysteroid disulfates eluted earlier than their 17α-counterparts and 20*S*-hydroxysteroid disulfates eluted earlier than their 20*R* counterparts. Regarding A ring derivatives, Δ^5 steroid disulfates eluted before

the fully reduced metabolites, the elution order of the reduced steroids being 3β,5β < 3β,5α < 3α,5β < 3α,5α. The chromatographic conditions were also able to separate the two estriol glucuronide isomers i.e. the 16-glucuronide and 3-glucuronide. Unfortunately, sulfate and glucuronide conjugates of two useful steroids in PORD diagnosis, androsterone and etiocholanolone, could not be separated under the selected conditions even after increasing the gradient to 1 h (Figure 1B).

Application to prenatal detection of disorders affecting estriol synthesis

We report preliminary studies to determine whether steroid disulfates in urine can be useful markers in the prenatal detection of disorders affecting estriol synthesis; until now only monoconjugates had been used. The background to this study being that unconjugated serum E3 is frequently measured at mid-pregnancy as a marker for Down's syndrome as part of a test called triple- or quad- marker screening (Haddow, et al. 1994). If results are low the question remains as to the reason, and our original research was directed to diagnosis of Smith-Lemli-Opitz Syndrome (SLOS), the clinically most severe cause of low E3 (Shackleton, *et al*. 2007). These studies led to investigation of other causes such as STSD and PORD.

Diagnostic ratios are frequently employed in steroid metabolomics and E3 frequently used as denominator. Dominant E3 conjugates are 3- and 16-glucuronides (30% and 60%, respectively) with about 2.5% as monosulfate and estriol-3-glucuronide-16-sulfate (6.5%) (Tikkanen, *et al*. 1973). We assessed E3 excretion from the measurement of glucuronide and monosulfate conjugates (Table 1).

Steroid sulfatase deficiency (STSD) (OMIM , 308100, location, Xp22.31)

This X-linked disorder prevents the release of steroid from steroid sulfates. A summary of the biosynthetic pathway leading to estriol is shown in Figure 2, illustrating that inactivity of the enzyme in placenta prevents 16αOHDHEA-S conversion to E3. This fetal 16αOHDHEA-S, androst-5-ene-3β,16α,17β-triol sulfate (5AT-S) and other steroid sulfates pass through the placenta and mother to be excreted in urine largely unchanged (Taylor and Shackleton, 1979).

STSD urine samples (N=11) and 11 controls were analyzed using the CIL scan method for disulfates complemented with the acquisition of 16αOHDHEA-S and E3 monoconjugates (Table 1). Among the disulfates measured by the CIL method, we found that the response ratio between six of them, namely 16αOHDHEA-diS, 5AD(17α)-diS, 5AD(17β)-diS, 5PT-diS, 21OHPreg-diS and 5PD-diS against E3 glucuronide (measured as sum of 3- and 16-glucuronides) was markedly increased in STSD.

Representative chromatograms of a normal pregnancy urine and one with an STSD affected fetus are shown in Figure 3. The ratio values for our normal and STSD data sets are shown in Figure 4A and show all analytes clearly distinguish STSD from normal. Additionally, we used the ratios to evaluate the relative efficacy of each analyte in diagnosis. The best steroid discriminatory ratio would show greatest difference between the lowest steroid sulfate/E3-G ratio value in STSD, and the highest ratio found in controls (Figure 4B). Interestingly, the ratios that gave the greatest differential were Δ^5 pregnenes; 5PD-diS, 5PT-diS and 21OHPreg-diS, not the C_{19} steroid sulfates on the direct biosynthetic pathway to E3. Combining ratio data can give an even greater separation for normal and affected; note the combined data for 21OHPreg-diS and 5PD-diS in Figure 4C. Such pregnene metabolites should be incorporated in MS based methodologies for detection of the disorder.

P450 oxido-reductase deficiency (PORD) (POR OMIM 124015 location: 7q11.23)

Several pregnant women carrying PORD fetuses have been studied by GC-MS (Shackleton, et al. 2004; Reisch, et al. 2013), but for only two were samples available for this study. Shackleton and co-workers (2004a) deduced that the dominant "feto-placental" maternal urinary steroid in PORD pregnancies was 3β5αPD-diS. While this steroid is also present in the disulfate fraction of normal pregnancy urine it is in much greater amount in PORD affected pregnancies. From its dominance together with reduced E3, it was concluded that this metabolite is a maternal excretory product of fetal pregnenolone. An intermediate precursor would be fetal steroid 5PD-diS. Excess pregnenolone and its sulfate are the result of an apparent "block" in 17-hydroxylase/C17-20 lyase secondary to attenuated POR activity (Figure 5). This block, together with suppressed 16α-hydroxylase (also due to PORD) causes reduction of fetal 16αOHDHEA-S production leading to low maternal E3 production and excretion. The precise sequence of reactions from fetal pregnenolone to 3β5αPD-diS, and localities of the conversions (fetal adrenal, liver, placenta and mother) is yet to be determined. The process is multi-step, probably including placental 3β-desulfation and likely 3β-hydroxysteroid dehydrogenase/isomerase. It has long been known that both 3β5αPD-diS and 5PD-diS are prominent disulfates in umbilical cord blood (Laatikainen, et al. 1972) so are freely synthesized and transported in the feto-placental unit. The synthetic sequence for pregnenolone conversion to urinary metabolites in normal and PORD affected pregnancies and neonate are shown in Figure 5. Evidence suggests the corresponding conversion of pregnenolone sulfate to DHEA-S is not an available pathway (Neunzig, et al. 2014; Sanchez-Guijo, et al. 2016; Rege, et al. 2017).

Besides the increased excretion of 3β5αPD-diS, we also observed an increase in the transitions corresponding to 5PD-diS and 3β,21-dihydroxy-5α-pregnan-20-one disulfate (21OHPreg3β5α-diS), the latter in spite of a likely POR requirement by fetal 21-hydroxylase. However, it should be noted that this fetal enzyme differs from that

coded by CYP21A2 required in cortisol synthesis (Guerami et al., 1988, Corsan, Macdonald and Casey., 1997).

In Figure 6 we illustrate the chromatographic profiles of the 3β5αPD-diS, 5PD-diS and 21OHPreg3β5α-diS and the E3 conjugates in a control (Figure 6A) and affected pregnancy (Figure 6B). The dominance of the 3β5αPD-diS in the affected pregnancies is striking. In GC-MS diagnosis of PORD prenatally the ratio of 3β5αPD/E3 was used, i.e. the ratio of principal PORD fetal metabolite to E3, the conventional feto/maternal metabolite. In Figure 6C are shown ratios for intact conjugates in PORD and controls. For the denominator (E3) we summed the total of both glucuronylated forms.

One of the GC-MS prenatal diagnostic ratios for PORD remains a challenge for LC-MS/MS under conditions developed for this study. With fetal PORD there is increased androsterone production as a result of the "alternative pathway" activity (Arlt, et al. 2004) resulting in markedly increased androsterone/etiocholanolone ratio (Shackleton et al 2004a). That ratio should theoretically be determined by direct analysis of glucuronides and this separation has been already reported by C18 columns both in glucuronides (Pozo et al. 2008) and unconjugated (Marcos and Pozo, 2016). Unfortunately, under current chromatographic conditions developed for the disulfates such isobaric monoconjugates (sulfates or glucuronides) could not be resolved.

Postnatal detection of PORD:

While this paper has focused on prenatal diagnosis of PORD by 3β5αPD-diS measurement, Shackleton and co-workers (Shackleton et al. 2004b) have shown that its precursor 5PD-diS is a key analyte in diagnosing the condition in the first months of life suggested its inclusion here. In PORD neonatal samples this steroid is

dominant, excretory values exceeding the classical major metabolites such as 16αOHDHEA-S and 16α-hydroxypregnenolone sulfate whose biosynthesis by 16α-hydroxylation is also POR dependent. In the first weeks of life the fetal zone of the adrenal is still dominant, but diminishing, and is responsible for producing a large amount of 3β-OH-Δ^5 steroids.

Figure 7 illustrates the separation of steroid disulfates in an affected PORD infant and normal infant. We have included 16αOHDHEA-S as analyte to act as denominator for a potential diagnostic ratio 5PD-diS/16αOHDHEA-S. This ratio is shown for three affected infants and normal controls in Figure 7C, clearly defining the condition. Interestingly, one of the first steroid disulfates to be identified in the neonatal period were 5AD(17α and 17β)-diS (Shackleton, et al. 1968a, Laatikainen, et al. 1972), and 16βOHDHEA-diS (Shackleton, et al. 1968b, Laatikainen, et al. 1972) and these are clearly separated with this methodology (Figure 7).

Smith-Lemli-Opitz Syndrome. "7-dehydrosterol reductase" deficiency. (SLOS) (OMIM 602858 *location: 11q13,4)*

This condition is caused by deficiency in 7-dehydrosterol reductase and the notable feature is a build-up of 7-and 8-dehydrocholesterol, which can be used to diagnose the condition when measured in amniotic fluid (Kelley,1994). The affected fetus can use these sterols as steroid precursors, resulting in the appearance in maternal urine of dehydro (DH) versions of common natural steroids. For instance, 5β-pregn-7(and 8)-ene-3α,17α,*20S*-triol, (7(8)-DHPT) and an estriol equivalent, principally 8-dehydroestriol (8-DHE3) (Guo, et al. 2001, Shackleton, et al. 1999). The biosynthesis of steroids in SLOS pregnancy is illustrated in Figure 8.

SLOS steroids are mainly excreted as glucuronides. Thus, distinct peaks corresponding to different isomers of 8-DHE3-G were found in all SLOS samples (Figure 9). On the other hand, the detection of 7(8)-DHPT-G provided more

difficulties due to endogenous interferences probably coming from other pregnenetriols and DH-hydroxypregnenolones which would share the same transition (Figure 9).

A systematic study has not been made of steroid sulfates in this condition and lack of appropriate authentic compounds has meant that only *candidate* chromatographic peaks were provisionally identified. Such peaks were chosen by having the expected CIL transitions for steroids with additional unsaturation and to be accepted as SLOS-specific "candidate" analytes these peaks had to be present in all six confirmed SLOS pregnancies, and be absent from controls. Two main metabolites were found. Peaks with the expected transitions for DHPT-diS and DHPD-diS were observed in all SLOS samples. Additionally, other minor metabolites such as DH-androstenediol-diS were also found. In Figure 9 the chromatograms of the proposed steroid disulfate analytes with DH-pregnanetriol glucuronide and DHE3-glucuronide are shown. Little information can be stated on stereochemistry of candidate analytes; not only that of 3- and 5-positions but both Δ^7 and Δ^8 isomers are likely present. The chromatograms illustrated were from one affected pregnancy and one control. Similar chromatograms were produced for the other five affected pregnancies and controls. Clearly this is the most challenging of the three conditions for conjugate LC-MS/MS analysis although aberrant steroid conjugate peaks definitive for SLOS were detected.

Distinguishing the disorders: summary

This study has focused on the mass spectrometric analysis of steroid disulfates, but steroid monosulfates and glucuronides have been included where required to determine ratios used for diagnosis. In order to evaluate the potential of the approach based on the combined screening of glucuronides, monosulfates and disulfates, we propose a panel of markers able to differentiate between the selected

disorders and control samples. We found that using the ratios 16αOHDHEA-S/E3-G, 3β5αPD-diS/E3-G, 5PD-diS/16αOHDHEA-S, 21OHPreg-diS/E3-G, 21OHPreg-diS/16αOHDHEA-S, 8DHE3-G/E3-G and DHPT-diS/E3-G allowed for the successful differentiation between the controls and the different disorders
Hopefully the study emphasizes the potential of LC-MS analysis of all conjugate types in future development of steroid metabolomics.

General discussion

The steroid disulfates are a minor fraction of the urinary steroid metabolome, but may provide significant markers of aberrant steroid biosynthesis. As a family, intact steroid disulfates have not been recently subject to detailed study due a lack of suitable analytical methodology. In the past, studyingthis family always involved time-consuming fractionation of free and conjugate families followed by solvolysis and GC-MS analysis. Most of the available literature stems from the 60's and 70's and it was shown early which secondary positions (assuming the primary sulfated position is the 3-hydroxyl) could be sulfated. These were 17 (α-and β), 16β- and 18 in C_{19} steroids and 20*S*- and 21- in C_{21} steroids. During that early research period the dominant biological materials chosen to study were associated with pregnancy. In that respect our current studies have followed this lead and the major disulfate components reported here were also noted in the early publications (Shackleton, et al. 1968a; Shackleton, et al. 1968b; Jänne, et al. 1969; Jänne and Vihko 1970; Laatikainen et al 1972; Meng and Sjövall 1997).

There is little definitive evidence as to which sulfotransferases are responsible for the secondary sulfation (Mueller et al 2015), and how disulfates are transported (Grosser et al 2017). Available sulfation evidence points solely to SULT2A1 which appears to have an active site capable of encompassing a wide variety of steroid substrates (both free and monosulfated) and conduct sulfation at either end of the

steroid molecule. Thus, it can sulfate free steroids or steroid monosulfates (Cook, et al. 2009).

A question remains as to whether disulfation is purely a catabolic reaction or if such steroids could be transportable reservoirs of active hormone precursors, as is likely the case for DHEA and estrone sulfates. Guerami and co-workers (1988) have proposed that 21OHPreg-diS is an 11-deoxycorticosterone (DOC) precursor during pregnancy, particularly since circulating levels of this mineralocorticoid and its sulfate are increased during gestation (Corsan, Macdonald and Casey 1997). It is known that the placenta is capable of hydrolyzing 21-sulfates and the enzyme responsible is the usual STS as 21-desulfation does not occur in STSD (Guerami, et al. 1988). Another possible reservoir for disulfates is 5AD(17β)-diS, potentially a testosterone or estradiol precursor. This steroid is also subject to STS action in mammals. In contrast, it is believed that human sulfatases are inactive on 17α- (C_{19} steroids) or 20*S*--sulfates, a situation shared with the commercial snail and mollusk enzymes used for hydrolysis in steroid analysis (Stevenson, et al. 2014).

In summary, we have provided analytical data on the steroid disulfates through their measurement as intact molecules by LC-MS/MS, employing CIL scan monitoring. We have attempted to use these additional members of the steroid metabolome to distinguish fetal disorders of steroid synthesis. To the best of our knowledge, this is the first time that direct analysis of steroid disulfates has proved its value for clinical diagnosis.

The ultimate goal of these studies is the ability to quantify the whole urinary steroid metabolome as unhydrolyzed conjugates, the monosulfates, disulfates, glucuronides and mixed sulfate-glucuronide conjugates. Studies of the plasma steroid metabolome should also be included. To achieve this goal will require the synthesis of a multitude of authentic steroids including appropriate internal standards and an improvement in chromatographic resolution.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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FIGURE LEGENDS

Figure 1

Chromatographic separation obtained for (A) seven synthesized isomers of pregnanediol disulfates and (B) androsterone and etiocholanolone glucuronide. Note that the method optimized for the separation of isomeric disulfate metabolites was not able to separate epimeric glucuronides.

Figure 2

Steroid synthesis in STSD pregnancy starting from fetal adrenal pregnenolone. Inactivity of STS prevents conversion of 16αOHDHEA-S to estriol in placenta so the former (and its metabolites) is excreted as sulfates by mother. C_{21} steroid sulfates upstream from 16αOHDHEA-S also pass the placenta without de-sulfation and are directly excreted in maternal urine.

Figure 3

Selected urinary Δ^5 steroid sulfate and estriol conjugate analysis in a control and STSD affected pregnancy. Note the markedly increased Δ^5 steroid mono and disulfates in STSD. Regarding the estriol conjugates it must be noted that glucuronides give lower MS transition responses than sulfates under the reported conditions. In reality, E3-S is a minor metabolite of estriol compared to the 3- and 16-glucuronides, although it appears contrary in the chromatograms.

Figure 4

Panel A: Ratios of steroid sulfates to E3 glucuronide (3+16) in STSD. The scale represents the ratios of raw peak areas of transitions, not the actual

amount quantified. All ratios show separation of controls from affected pregnancies. Panel B: a measure of the difference between the lowest STSD ratio and highest control ratio. The higher this value, the greater the separation between affected and normal. Two Δ^5 pregnene di-sulfates are the most efficacious diagnostic analytes. Panel C: combining data from 5PD-diS and 21OHPreg-diS increases discrimination between normal and STSD.

Figure 5

Steroid biosynthesis and metabolism in PORD and normal pregnancies and neonates. *Normal pregnancy:* fetal adrenal pregnenolone is converted to maternally excreted estriol conjugates (POR essential). *PORD pregnancy*: excess adrenal pregnenolone (due to PORD) is metabolized primarily to maternally excreted 3β5αPD-diS. *Normal neonate*: Excretion product 16αOHDHEA-S and other sulfates. *PORD neonate*: major pregnenolone excretory product 5PD-diS.

Figure 6

Selected Reaction Monitoring (SRM) transition chromatograms of estriol conjugates and steroid disulfates in control (A) and PORD affected pregnancies (B). Note high excretion of 3β5αPD-diS and 21OHPreg3β5α-diS. (C) Graph shows peak area ratios (analyte/E3-G) for 2 affected pregnancies and 11 controls.

Figure 7.

SRM chromatograms of control (A) and affected PORD babies (B). The key analyte is the pregnenolone metabolite 5PD-diS and its relative excess is determined by peak area ratio to 16αOHDHEA-S, normally a dominant metabolite in neonates. Discrimination obtained by the use of 5PD-

diS/16αOHDHEA-S (C) and 21OHPreg-diS/16αOHDHEA-S (D) between healthy and PORD babies.

Figure 8.

Deficiency of 7-dehydrosterol reductase (DHCR7, SLOS] causes 7-or 8-dehydro-cholesterol to be used as fetal precursor for downstream steroids which retain B- ring unsaturation. Dehydro-pregnanetriol (DHPT) and dehydroestriol (DHE3) glucuronides have been used classically for diagnosis but here are candidate disulfates tentatively identified, compounds not seen in controls.

Figure 9.

SRM chromatograms of candidate analytes in SLOS pregnancy. (A) control pregnancy and (B) affected pregnancy. Transition chromatograms for known diagnostic steroid glucuronides and candidate disulfates useful for diagnosis. While authentic steroids are not available, these steroids, with appropriate transitions were only present in affected pregnancies. Steroid A/B ring stereochemistry including Δ7/Δ8 unsaturation is as yet unknown. In the control chromatograms E3 conjugates are shown but all SLOS candidate disulfates and glucuronides are absent.

Table 1. SRM parameters of selected steroids

Analyte	Disorder	MW	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Monoconjugates						
E3-3G	all	464	2.3	463	287	30
E3-16G	all	464	9.3	463	287	30
E3-3S	all	368	7.3	367	287	35
16OHDHEA-S	STSD/PORD*	384	16.0	383	97	40
DHE3-G	SLOS	462	8.6/9.1	461	285	30
DHPT-G	SLOS	510	21.3	509	75	30
Disulfates						
5AD(17α)-diS	STSD	450	15.5	224	351	15
5AD(17β)-diS	STSD	450	13.4	224	351	15
16αOHDHEA-diS	STSD	464	10.0	231	365	15
16βOHDHEA-diS	STSD	464	8.2	231	365	15
5PT-diS	STSD	494	10.5	246	395	15
21OHPreg-diS	STSD/PORD	492	18.2	245	393	15
5PD-diS	STSD/PORD*	478	16.7	238	379	15
3β5αPD-diS	PORD	480	17.6	239	381	15
DH5AD-diS	SLOS	448	11.4	223	349	15
DHPT-diS	SLOS	494	12.6	246	395	15
DHPD-diS	SLOS	478	15.6	238	379	15

* PORD neonatal

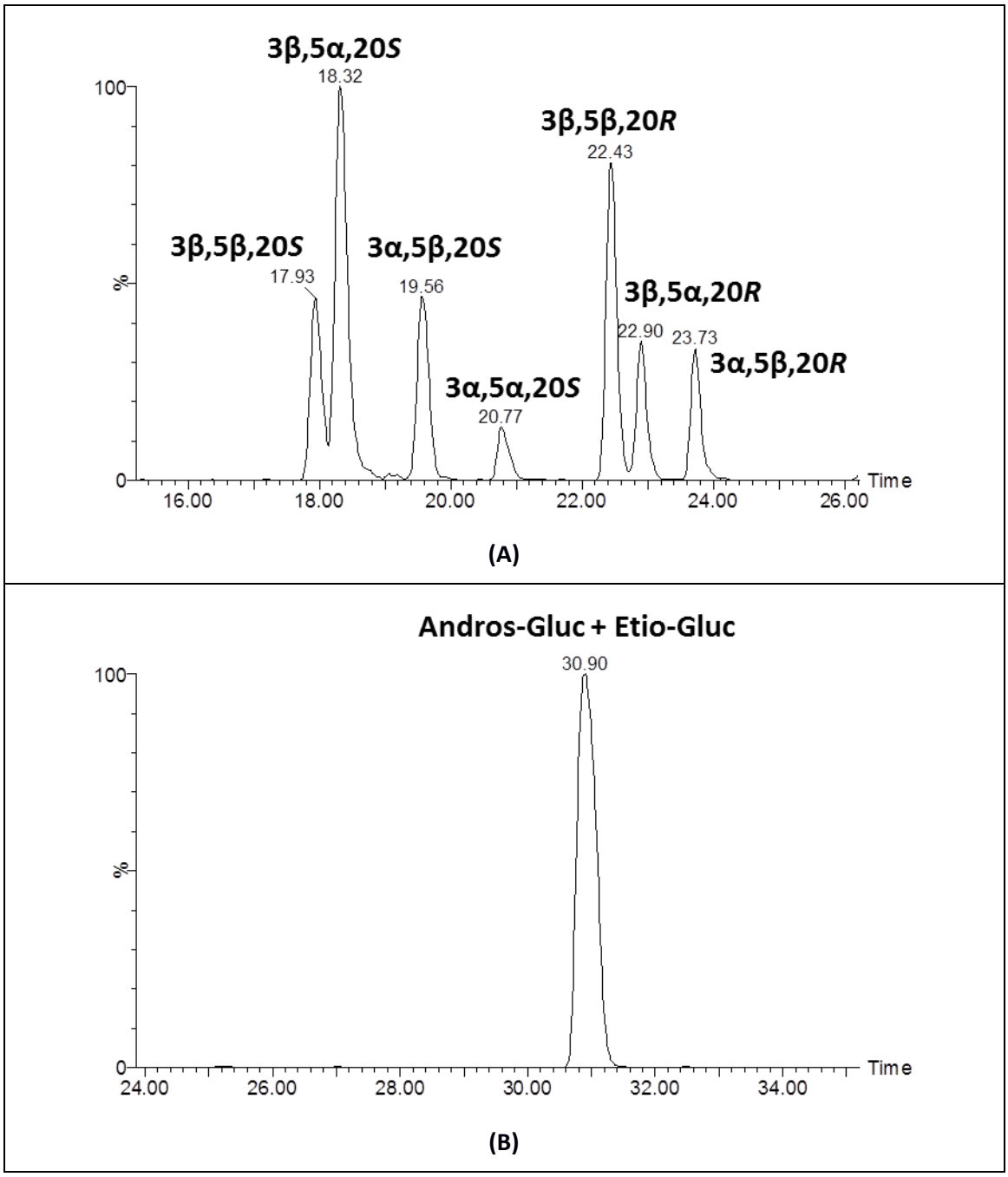

3β,5α,20S
18.32
3β,5β,20R
22.43
3β,5β,20S
17.93
3α,5β,20S
19.56
3β,5α,20R
22.90
23.73
3α,5β,20R
3α,5α,20S
20.77
100
%
0
16.00
18.00
20.00
22.00
24.00
26.00
Time
(A)
Andros-Gluc + Etio-Gluc
30.90
100
%
0
24.00
26.00
28.00
30.00
32.00
34.00
Time
(B)

Figure 1

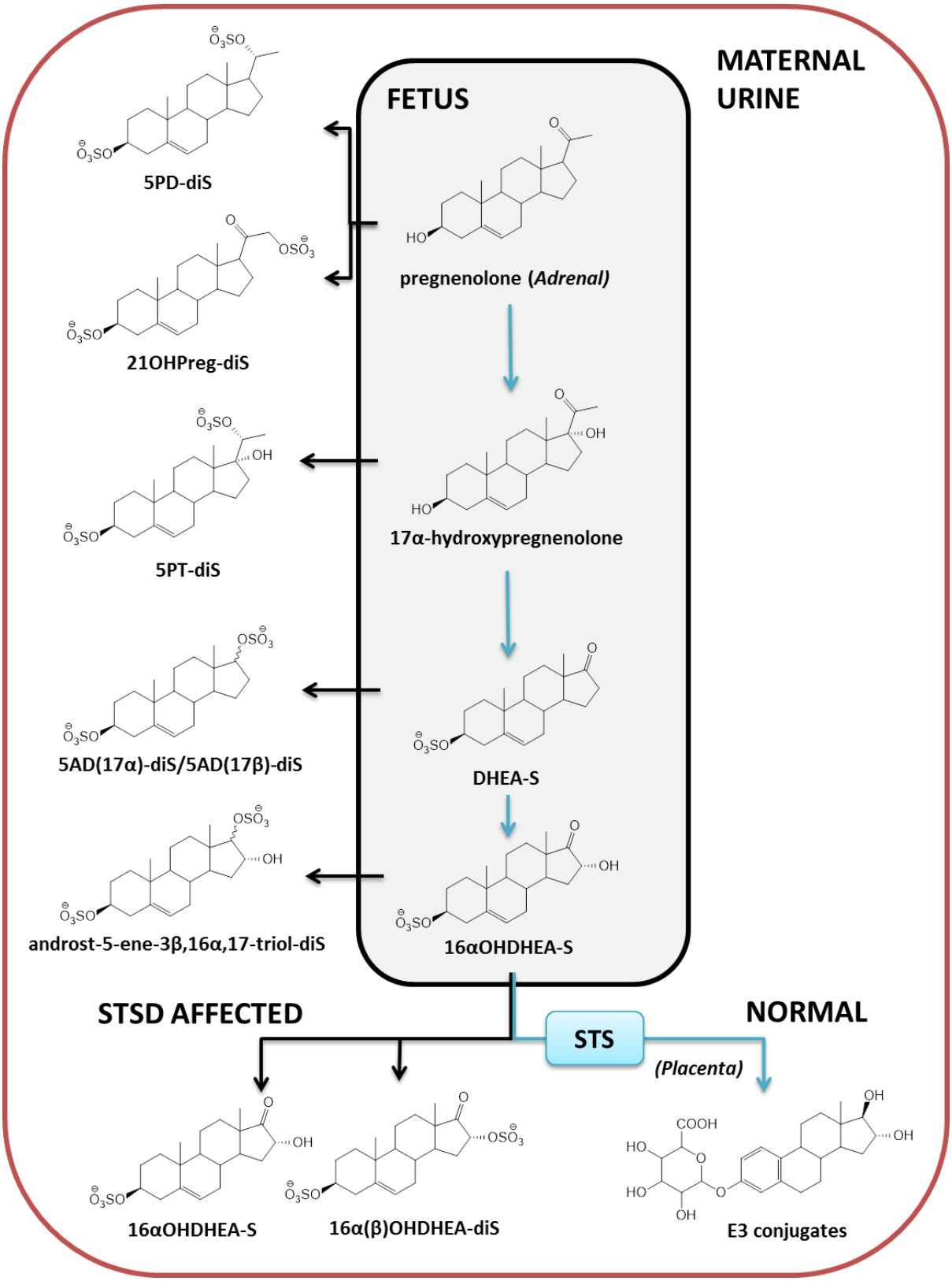

MATERNAL URINE
FETUS
5PD-diS
pregnenolone (Adrenal)
21OHPreg-diS
17α-hydroxypregnenolone
5PT-diS
DHEA-S
5AD(17α)-diS/5AD(17β)-diS
16αOHDHEA-S
androst-5-ene-3β,16α,17-triol-diS
STSD AFFECTED
STS
NORMAL
(Placenta)
16αOHDHEA-S
16α(β)OHDHEA-diS
E3 conjugates

Figure 2

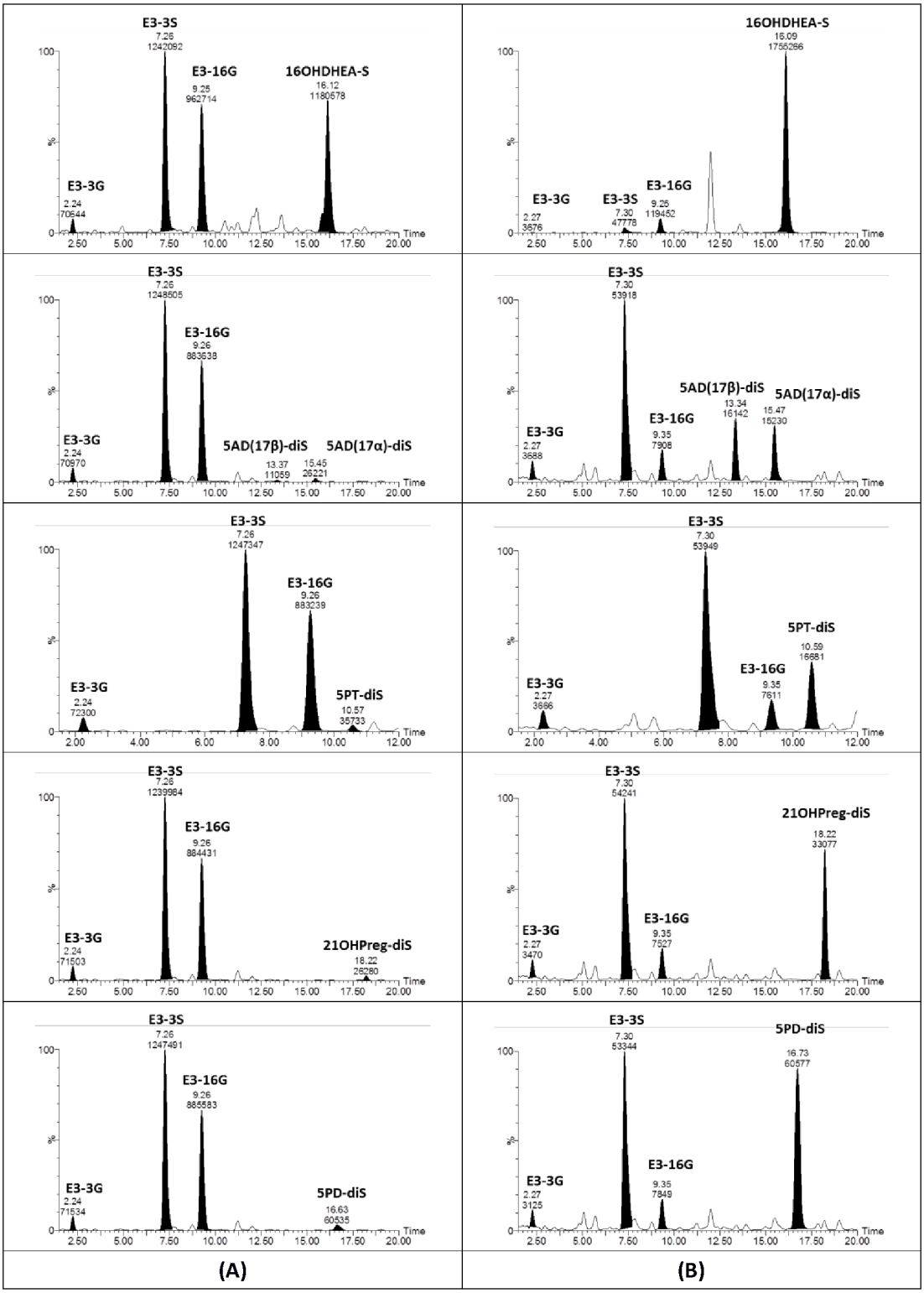

E3-3S
7.26
1242092
E3-16G
9.25
962714
16OHDHEA-S
16.12
1180678
E3-3G
2.24
70544
16OHDHEA-S
16.09
1755286
E3-3G
2.27
3676
E3-3S
7.30
47778
E3-16G
9.25
119452
E3-3S
7.26
1248505
E3-16G
9.26
883638
E3-3G
2.24
70970
5AD(17β)-diS
13.37
11059
5AD(17α)-diS
15.45
26221
E3-3S
7.30
53918
5AD(17β)-diS
13.34
16142
5AD(17α)-diS
15.47
15230
E3-16G
9.35
7908
E3-3G
2.27
3688
E3-3S
7.26
1247347
E3-16G
9.26
883239
E3-3G
2.24
72300
5PT-diS
10.57
35733
E3-3S
7.30
53949
5PT-diS
10.59
16681
E3-16G
9.35
7611
E3-3G
2.27
3686
E3-3S
7.26
1239984
E3-16G
9.26
884431
E3-3G
2.24
71503
21OHPreg-diS
18.22
26280
E3-3S
7.30
54241
21OHPreg-diS
18.22
33077
E3-16G
9.35
7527
E3-3G
2.27
3470
E3-3S
7.26
1247491
E3-16G
9.26
885583
E3-3G
2.24
71534
5PD-diS
16.63
60535
E3-3S
7.30
53344
5PD-diS
16.73
60577
E3-16G
9.35
7849
E3-3G
2.27
3125
(A)
(B)

Figure 3

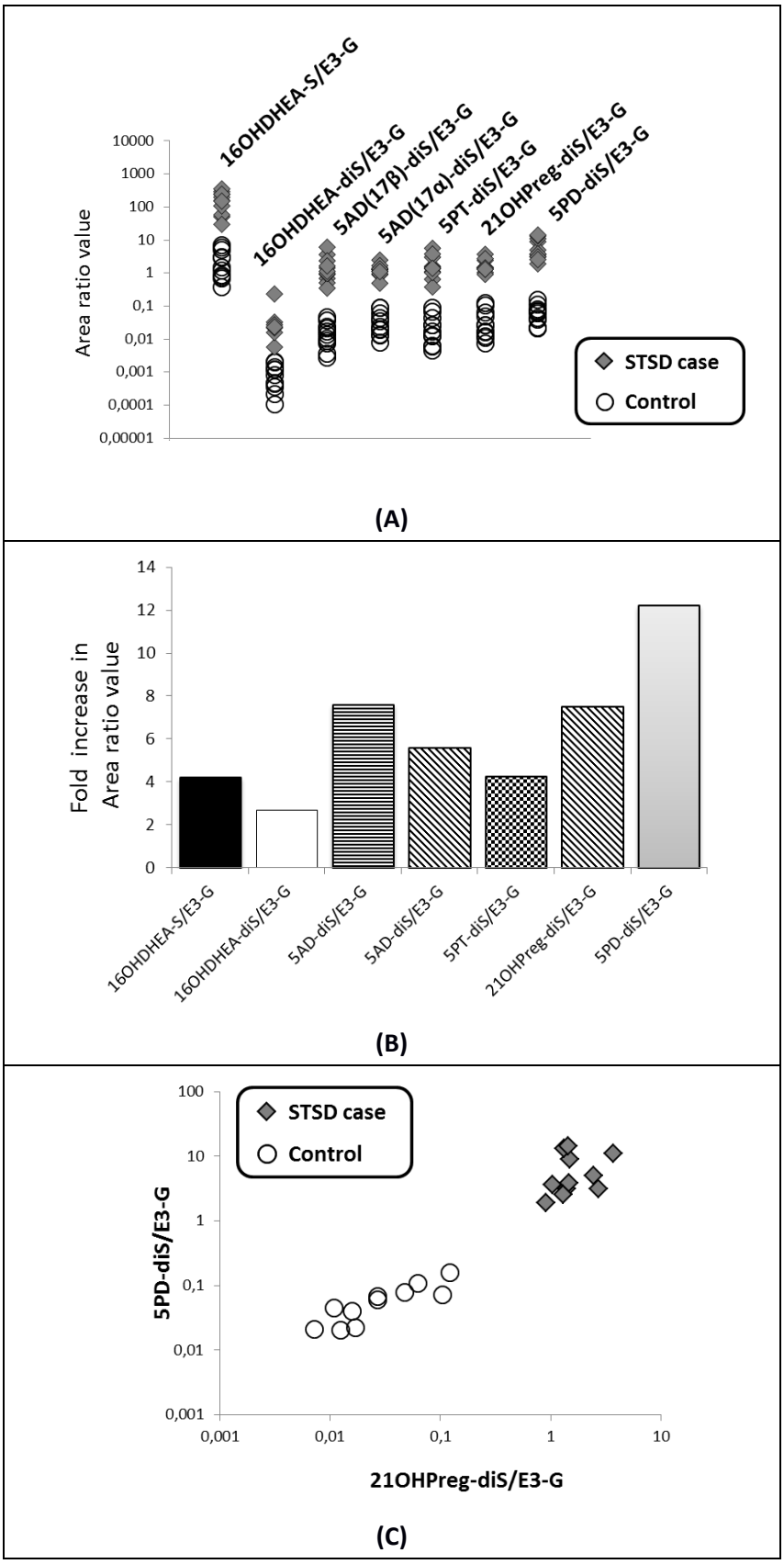

16OHDHEA-S/E3-G
16OHDHEA-diS/E3-G
5AD(17β)-diS/E3-G
5AD(17α)-diS/E3-G
5PT-diS/E3-G
21OHPreg-diS/E3-G
5PD-diS/E3-G
Area ratio value
10000
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
STSD case
Control
(A)
Fold increase in Area ratio value
14
12
10
8
6
4
2
0
16OHDHEA-S/E3-G
16OHDHEA-diS/E3-G
5AD-diS/E3-G
5AD-diS/E3-G
5PT-diS/E3-G
21OHPreg-diS/E3-G
5PD-diS/E3-G
(B)
100
10
1
0,1
0,01
0,001
5PD-diS/E3-G
STSD case
Control
0,001
0,01
0,1
1
10
21OHPreg-diS/E3-G
(C)

Figure 4

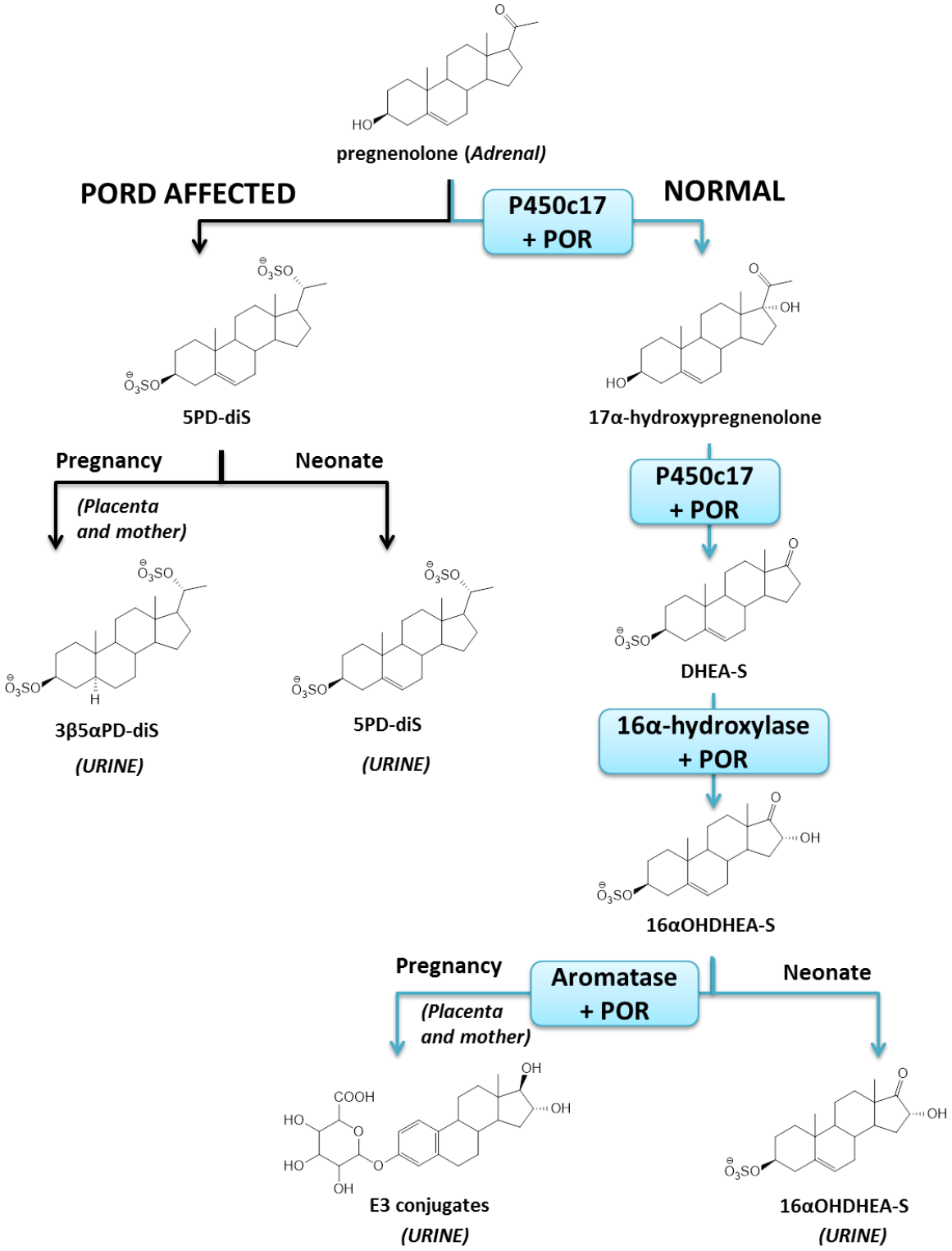

pregnenolone (Adrenal)
PORD AFFECTED
P450c17 + POR
NORMAL
5PD-diS
17α-hydroxypregnenolone
Pregnancy
Neonate
(Placenta and mother)
P450c17 + POR
DHEA-S
3β5αPD-diS
(URINE)
5PD-diS
(URINE)
16α-hydroxylase + POR
16αOHDHEA-S
Pregnancy
Aromatase + POR
Neonate
(Placenta and mother)
E3 conjugates
(URINE)
16αOHDHEA-S
(URINE)

Figure 5

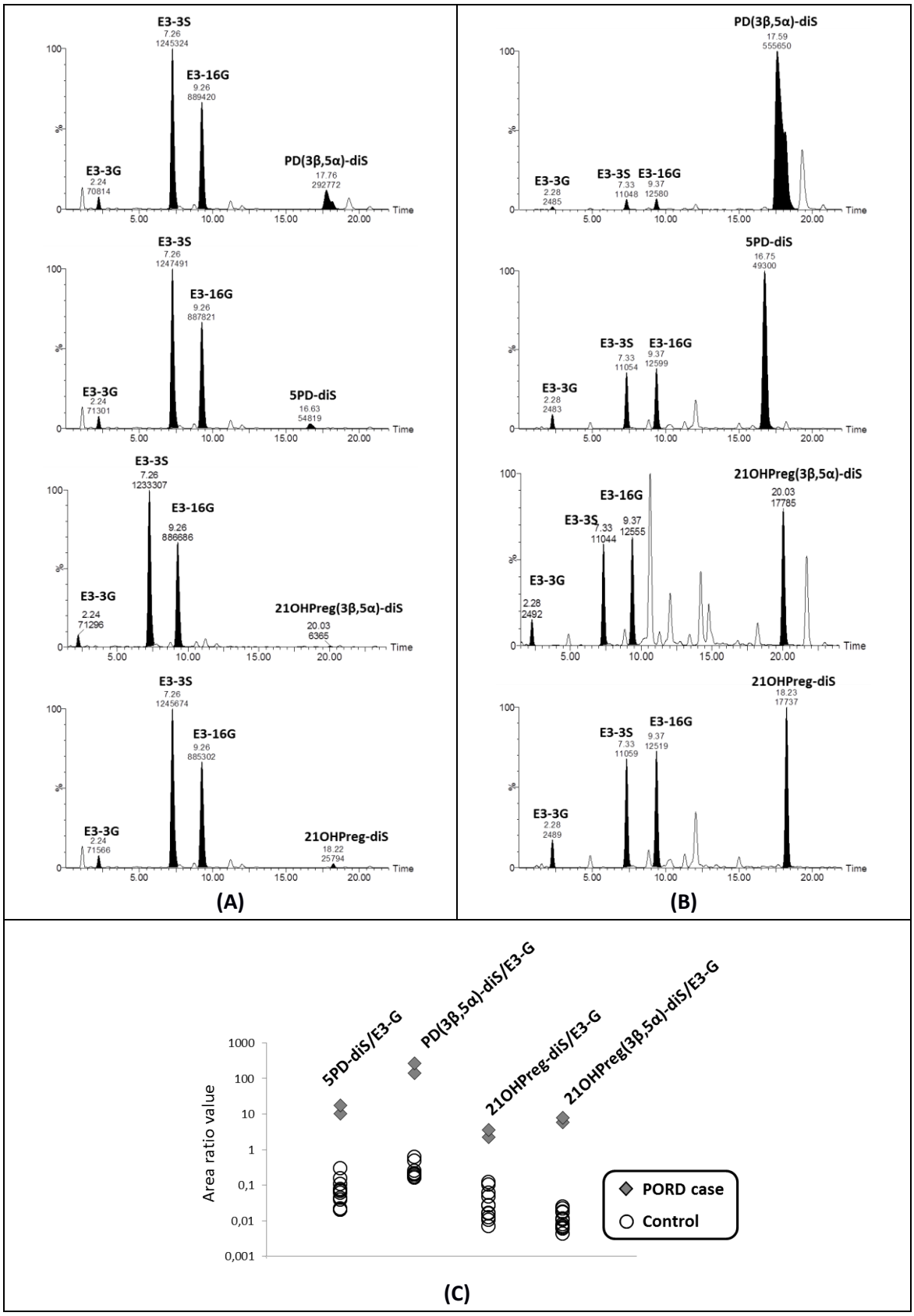
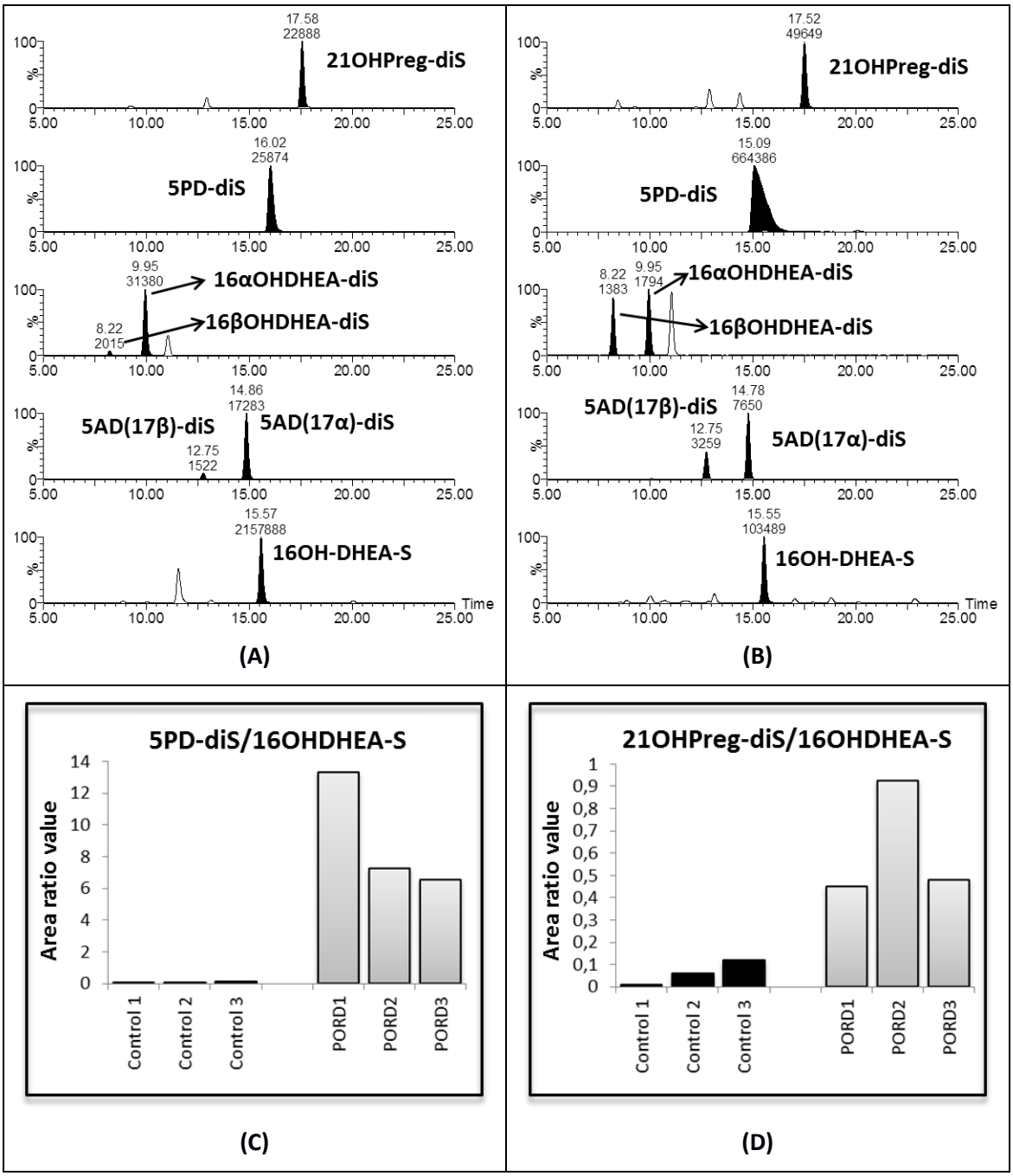

E3-3S
7.26
1245324
E3-16G
9.26
889420
E3-3G
2.24
70814
PD(3β,5α)-diS
17.76
292772
Time
5.00
10.00
15.00
20.00
100
%
0
E3-3S
7.26
1247491
E3-16G
9.26
887821
E3-3G
2.24
71301
5PD-diS
16.63
54819
E3-3S
7.26
1233307
E3-16G
9.26
886686
E3-3G
2.24
71296
21OHPreg(3β,5α)-diS
20.03
6365
E3-3S
7.26
1245674
E3-16G
9.26
885302
E3-3G
2.24
71566
21OHPreg-diS
18.22
25794
(A)
PD(3β,5α)-diS
17.59
555650
E3-3G
2.28
2485
E3-3S
7.33
11048
E3-16G
9.37
12580
5PD-diS
16.75
49300
E3-3S
7.33
11054
E3-16G
9.37
12599
E3-3G
2.28
2483
21OHPreg(3β,5α)-diS
20.03
17785
E3-16G
E3-3S
7.33
11044
9.37
12555
E3-3G
2.28
2492
21OHPreg-diS
18.23
17737
E3-3S
7.33
11059
E3-16G
9.37
12519
E3-3G
2.28
2489
(B)
5PD-diS/E3-G
PD(3β,5α)-diS/E3-G
21OHPreg-diS/E3-G
21OHPreg(3β,5α)-diS/E3-G
Area ratio value
1000
100
10
1
0,1
0,01
0,001
PORD case
Control
(C)

Figure 6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22888
21OHPreg-diS
16.02
25874
5PD-diS
9.95
31380
16αOHDHEA-diS
8.22
2015
16βOHDHEA-diS
14.86
17283
5AD(17β)-diS
5AD(17α)-diS
12.75
1522
15.57
2157888
16OH-DHEA-S
Time
(A)
17.52
49649
21OHPreg-diS
15.09
664386
5PD-diS
8.22
1383
9.95
1794
16αOHDHEA-diS
16βOHDHEA-diS
5AD(17β)-diS
14.78
7650
12.75
3259
5AD(17α)-diS
15.55
103489
16OH-DHEA-S
Time
(B)
5PD-diS/16OHDHEA-S
Area ratio value
Control 1
Control 2
Control 3
PORD1
PORD2
PORD3
(C)
21OHPreg-diS/16OHDHEA-S
Area ratio value
Control 1
Control 2
Control 3
PORD1
PORD2
PORD3
(D)

Figure 7

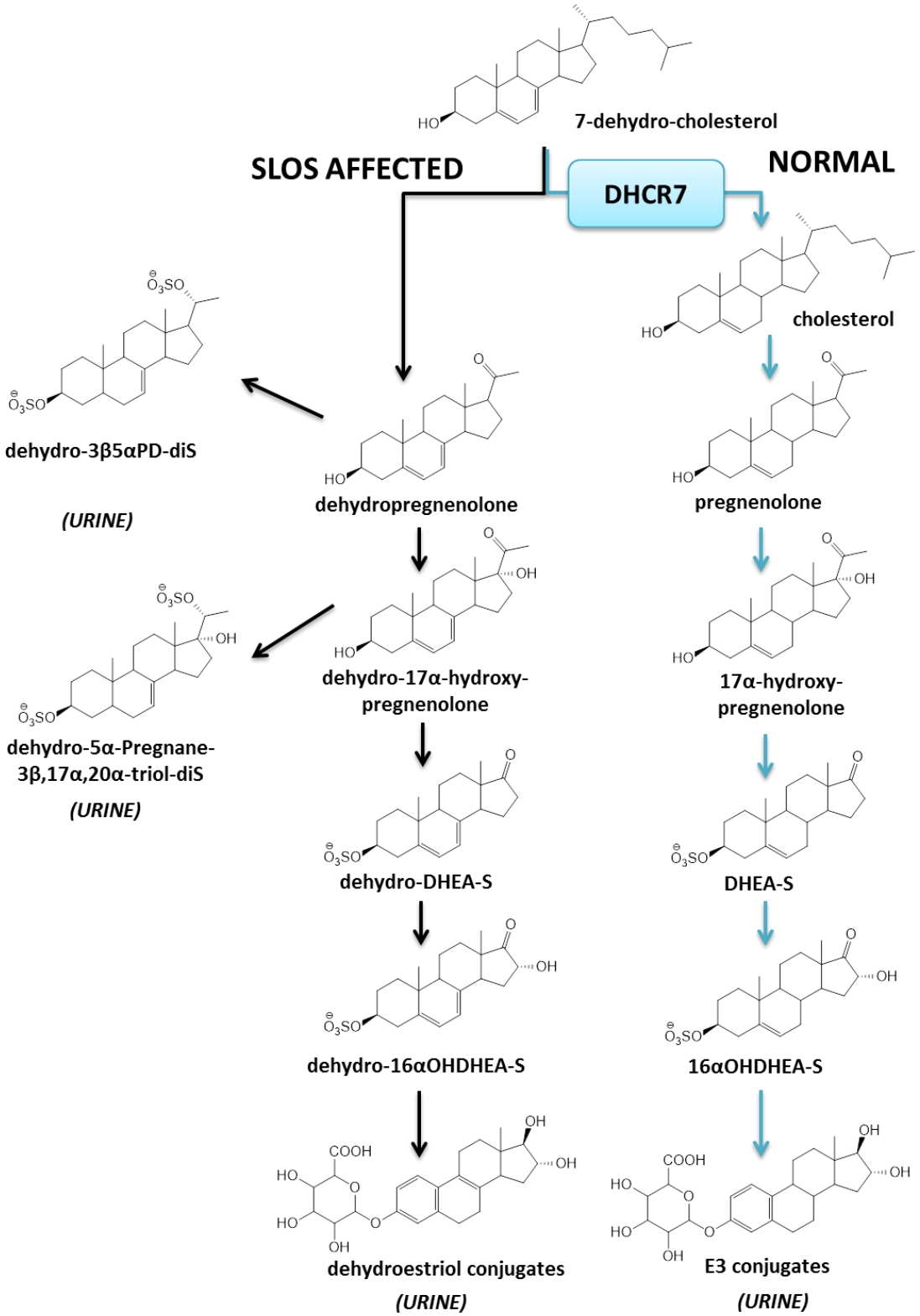
7-dehydro-cholesterol
SLOS AFFECTED
DHCR7
NORMAL
cholesterol
dehydro-3β5αPD-diS
(URINE)
dehydropregnenolone
pregnenolone
dehydro-17α-hydroxy-pregnenolone
17α-hydroxy-pregnenolone
dehydro-5α-Pregnane-3β,17α,20α-triol-diS
(URINE)
dehydro-DHEA-S
DHEA-S
dehydro-16αOHDHEA-S
16αOHDHEA-S
dehydroestriol conjugates
(URINE)
E3 conjugates
(URINE)

Figure 8

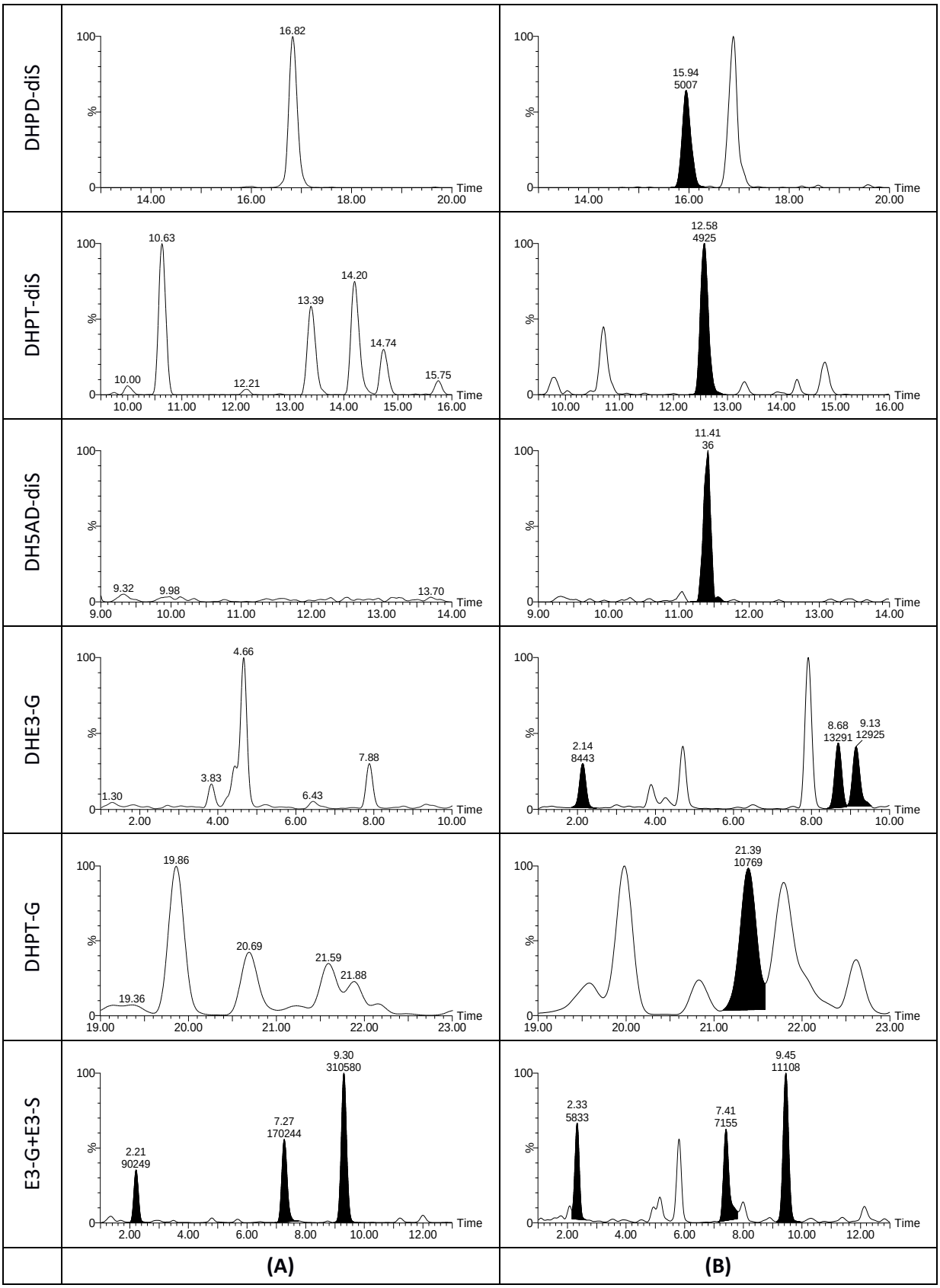
DHPD-diS
16.82
15.94
5007
DHPT-diS
10.63
14.20
13.39
14.74
10.00
12.21
15.75
12.58
4925
DH5AD-diS
9.32
9.98
13.70
11.41
36
DHE3-G
4.66
3.83
1.30
6.43
7.88
2.14
8443
8.68
13291
9.13
12925
DHPT-G
19.86
20.69
21.59
21.88
19.36
21.39
10769
E3-G+E3-S
9.30
310580
7.27
170244
2.21
90249
2.33
5833
7.41
7155
9.45
11108
Time
%
(A)
(B)

Figure 9.